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SEPTEMBER, 1918

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C.-E. A. WINSLOW

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A SYNTHETIC MEDIUM FOR THE DIRECT ENUMERATION OF ORGANISMS OF THE COLON-AEROGENES GROUP

S. HENRY AYERS AND PHILIP RUPP

From the Research Laboratories of the Dairy Division, United States Department of Agriculture

Received for publication June 12, 1918

The direct numerical determination of the organisms of the colon-aerogenes group has attracted the attention of numerous investigators and several different media have been originated for this purpose. There are many difficulties which are encountered in an attempt to devise a selective medium upon which organisms of this group will grow while other species of bacteria are excluded. The difficulties are too well recognized by all who have attempted direct quantitative determinations to warrant further discussion.

The media commonly used for the direct determination of the colon-aerogenes group are very complex and depend largely for their success upon some constituent which inhibits the non-gas forming lactose fermenting organisms. This seems to be the wrong principle. Instead of depending upon inhibition of certain species it is believed that it is far better to simplify the medium to a point where the sources of nitrogen and carbon are in such a form as to allow only the growth of the organisms desired. With this view in mind a simple synthetic medium has been devised in which there is a single source of nitrogen, namely, sodium ammonium phosphate and a single source of carbon, namely, lactose.

COMPOSITION OF THE MEDIUM

Solution I

	per cent
Sodium ammonium phosphate	0.4
Acid potassium phosphate	0.2
Lactose	1.0

Dissolve in distilled water.

Solution II

Filtered solution of agar in distilled water..... 3.0

Mix solution I and II in equal proportions while hot and put up in definite amounts of 100 cc., or more, in flasks or bottles and then sterilize. The 3 per cent agar solution is made up separately and kept in stock merely for convenience. Agar can be added directly to solution I at the time of preparation if desired, using 1.5 per cent. A slight precipitate may appear upon sterilization, but this does not interfere with the count and may not appear on the plate.

INDICATOR

To indicate the acid colonies basic fuchsin is employed and decolorized by sodium sulfite using the following solutions:

Solution A. One per cent alcoholic solution of basic fuchsin.

Solution B. Five per cent aqueous solution sodium sulfite, freshly prepared.

At the time of plating, after the agar medium is melted and while at its highest temperature add for each 100 cc. of medium 0.5 cc. of a 1 per cent alcoholic basic fuchsin solution and follow at once with 0.5 cc. of a freshly prepared 5 per cent sodium sulfite solution. Mix thoroughly and allow to cool, then pour into plates as usual. The red color is not entirely destroyed by the sulfite and at time of plating the medium is pink.

Purified litmus or brom cresol purple can be used as indicators, but if more than about thirty colonies appear on the plate the entire medium changes to the acid color of the indicator and it then becomes impossible to detect acid colonies.

With decolorized basic fuchsin there is a red coloration or at least a deep red colony which is probably caused by the combined action of acid and aldehyde as has been shown by De Bord (1917). This indicator is preferred, therefore, to the two previously mentioned, because the acid-forming organisms of the colon-aerogenes group produce deep red colonies which can be detected when present in any countable number even when the medium has become entirely red.

INCUBATION

Plates may be incubated at 30°C. or at 37°C. and should be counted after forty-eight hours. The colonies appear somewhat more quickly and are larger at the higher temperatures. If the plates are incubated longer than forty-eight hours some bacteria which do not belong to the colon-aerogenes group may develop. Upon incubation the medium becomes red, but there is no difficulty in distinguishing the characteristic deep red colonies.

APPEARANCE OF COLONIES OF ORGANISMS OF THE COLON-AEROGENES GROUP

The colonies appear on the plates as medium sized red colonies with a deep red ring around them. The color about the colony is quite characteristic, although some have only a deep red color in the colony itself.

Bacteria which may also grow upon the medium and which do not belong to the colon-aerogenes group usually give pink or uncolored colonies which can be readily distinguished from those of the gas formers. Before this medium is used for routine work it is recommended that pure cultures of the organisms of the colon-aerogenes group be plated on the medium in order that the appearance of their colonies may be observed.

SELECTIVE ACTION OF THE MEDIUM

The synthetic medium has a selective instead of inhibitory action for two reasons. First, it allows only the development of bacteria which can obtain their nitrogen from a very simple compound, such as sodium ammonium phosphate. This naturally prevents the growth of a large number of species of bacteria. Second, the only source of carbon is lactose which therefore allows only the growth of bacteria which ferment this sugar.

A large number of organisms of the colon-aerogenes group have been tried and all found to be capable of utilizing nitrogen and carbon from the above mentioned sources. Relatively few other species of bacteria were encountered which could grow on

this medium and only occasionally were colonies of non-gas forming organisms observed which resembled those of organisms of the colon-aerogenes group.

Certain molds may develop on this medium which first appear as minute red colonies, but later they show the typical appearance of mold colonies.

It is not claimed that the medium is perfect in its selective action, that is to say, that it allows only the growth of organisms of the colon-aerogenes group. Probably a medium perfect in this respect can never be obtained. Every colony which develops can not be considered a gas former, but from the appearance of the colonies it is believed an accurate direct colon count can be obtained.

The flora of the material under examination may influence the accuracy of the count and the value of this simple synthetic medium. It has proven of great value in the determination of the colon count of milk and undoubtedly it can be used in the examination of other foods. A few results indicate that the medium should be particularly useful in the direct enumeration of organisms of the colon-aerogenes group in water.

This synthetic medium can probably be improved and it is hoped that it will be given a thorough trial in different laboratories and its defects brought to the attention of the writers.

ADVANTAGES OF THE MEDIUM

1. Apparent accuracy in the direct enumeration of bacteria of the colon-aerogenes group.
2. Constancy of composition.
3. Simplicity of preparation.
4. Cheapness.

REFERENCE

De Bord, George C. 1917 The fuchsin aldehyde reaction on endo medium. Jour. Bact, **2**, 309–314.

THE ENDO MEDIUM FOR THE ISOLATION OF B. DYSENTERIAE AND A DOUBLE SUGAR MEDIUM FOR THE DIFFERENTIATION OF B DYSENTERIAE, SHIGA AND FLEXNER[1]

I. J. KLIGLER AND J. DEFANDORFER

From the Laboratories of the Rockefeller Institute for Medical Research, New York

Received for publication December 21, 1917

CORRECTION

Through an error in proof reading the name of the junior author of the paper on "The Endo Medium for the Isolation of B. dysenteriae and a Double Sugar Medium for the Differentiation of B. dysenteriae, Shiga and Flexner," on page 437 of Volume III of the JOURNAL OF BACTERIOLOGY for September, 1918, was misspelled. The names of the authors should have appeared as follows: I. J. Kligler and J. H. Defandorf.

bacillus. Of the two available media for this purpose, litmus-lactose agar and Endo's medium, the latter presented certain advantages.

Modified Endo medium. In carrying forward the cultivation on the Endo medium it was discovered that pure cultures of the Shiga dysentery bacillus or cultures in fecal emulsions sometimes gave irregular results. It appeared at first that the fuchsin acted as the inhibitive agent, being more effective against the Shiga bacillus than against the Flexner group of bacilli. In following out this line of experiment, it was determined that the

[1] Work aided by a grant from the International Health Board of the Rockefeller Foundation.

this medium and only occasionally were colonies of non-gas forming organisms observed which resembled those of organisms of the colon-aerogenes group.

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THE ENDO MEDIUM FOR THE ISOLATION OF B. DYSENTERIAE AND A DOUBLE SUGAR MEDIUM FOR THE DIFFERENTIATION OF B DYSENTERIAE, SHIGA AND FLEXNER[1]

I. J. KLIGLER AND J. DEFANDORFER

From the Laboratories of the Rockefeller Institute for Medical Research, New York

Received for publication December 24, 1917

This paper is based on a study of means of isolating *Bacillus dysenteriae* from human excreta and polluted soil or water, in which it may be contained. Previous experience (Kligler, 1918) had shown that with respect to sensitiveness to chemicals, the dysenteric group of bacilli approached the Gram positive bacteria; or, in other words, departed from the typhoid and paratyphoid groups. On the other hand, when an enriching medium was employed for cultivation (nutrose, bile, egg), it proved just as favorable to the growth of other intestinal bacteria as to *B. dysenteriae*. Hence resort was had to a plate medium that would afford colony differentiation and permit at the same time the development of very delicate strains of the dysentery bacillus. Of the two available media for this purpose, litmus-lactose agar and Endo's medium, the latter presented certain advantages.

Modified Endo medium. In carrying forward the cultivation on the Endo medium it was discovered that pure cultures of the Shiga dysentery bacillus or cultures in fecal emulsions sometimes gave irregular results. It appeared at first that the fuchsin acted as the inhibitive agent, being more effective against the Shiga bacillus than against the Flexner group of bacilli. In following out this line of experiment, it was determined that the

[1] Work aided by a grant from the International Health Board of the Rockefeller Foundation.

addition either of nutrose or bile produced a marked improvement of the medium, the former surpassing the latter in this respect. Further study showed, however, that other factors were concerned, and finally, by elimination and new cultivation, a medium was evolved which offered distinct advantages for the cultivation of the dysentery bacilli. The modification consists in the substitution of sodium bisulphite as recommended by Robinson and Rettger (1916) and the accurate adjustment of the hydrogen ion concentration to a P_H value of 7.6–7.8. The medium as ordinarily prepared with a phenolphthalein reaction of +0.2 gives an end reaction, after the addition of the sodium-sulphite which is alkaline, ranging from P_H 8.4 to 8.8. This degree of alkalinity is inhibitive to the growth of the Shiga bacillus.

Either meat-infusion or beef-extract agar may be used. We use a beef-extract medium prepared as follows:

Peptone	10.00 grams
Beef extract	3.00 grams
NaCl	5.00 grams
Agar	15.00 grams
Water	1000 cc.

All the ingredients except the agar are dissolved first; the agar is then added and the mixture autoclaved for one hour at 15 pounds pressure. It is then cooled to 50°C., white of egg added (2 eggs to 5 liters), and steamed in the Arnold for thirty minutes. The reaction is then adjusted to P_H 7.4, with phenolsulphonephthalein as the indicator, the medium boiled on the free flame for six to seven minutes, filtered, flasked, and autoclaved.

This constitutes the stock[2] medium from which all the special media—brilliant green, Endo, etc.—may be prepared. Before pouring the Endo plates the reaction is adjusted to P_H 7.6–7.8, and the lactose and the fuchsin-sulphite are added in the usual manner. It has been our experience that about 0.8 cc. N NaOH

[2] This agar can be used for all purposes. The reaction is favorable for the growth of all common bacteria. In places where a large amount of medium is used, and in the field, it is especially advantageous to have a single stock that can serve as a basis for the various modified media.

was required for every 100 cc. of agar. It is necessary, however, to determine the exact amount for each fresh lot of agar.

Double sugar medium. Having developed the colonies on the Endo plate, it is necessary to carry the differentiation further so as to confirm the nature of the selected colonies. Russell (1911) makes use of a double sugar agar containing 0.1 per cent glucose and 1 per cent lactose. While this medium is effective, it does not permit the distinction of *B. typhosus* and the two classes—alkaline or Shiga and acid or Flexner, etc.—of dysentery bacilli from each other. We have substituted 0.5 per cent mannite for the lactose, with the effect of accomplishing the purpose.

The selected colonies are stabbed in the butt of the slanted tube and also streaked on the surface. Andrade's indicator is used. The differentiation is made after twenty-four hours' incubation at 37°C. The Shiga bacillus yields a slight reddening of the butt but no change of the surface. The acid class of dysentery bacilli and *B. typhosus* color the entire medium red; no gas is produced. *B. alcaligenes* produces no changes; while the paratyphoid bacilli cause both reddening and gas formation. *B. proteus*, on the other hand, produces gas but no reddening. If decolorized fuchsin is employed as indicator, then the paratyphoid bacilli A and B can be distinguished from each other, as the latter brings about final decolorization of the medium. However, the main advantage of this medium is that it permits of a more rapid separation of the two classes of dysenteric bacilli—the Shiga and the Flexner.

SUMMARY

A study of the Endo medium, as applied to *B. dysenteriae*, indicated that the most important single condition that must be carefully controlled, particularly if the medium is intended for the isolation of dysenteric bacilli, is the end reaction. The ingredients are of singificance in so far as they furnish the elements essential for the growth of the organisms. Given an otherwise favorable Endo medium, however, the Shiga bacillus will or will not grow, depending on whether the end reaction is P_H 7.6–7.8 or P_H 8.4–8.8, the reaction ordinarily obtained.

A mannite-glucose double sugar medium on the principle of Russell's double-sugar agar is described, to be employed for rapid differentiation of the Shiga and Flexner class of dysenteric bacilli.

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NOTE ON CROSS-AGGLUTINATION OF B. COLI COMMUNIS AND B. DYSENTERIAE SHIGA[1]

I. J. KLIGLER

From the Laboratories of the Rockefeller Institute for Medical Research, New York

Received for publication December 24, 1917

This note refers to an instance of cross-agglutination between two distinct bacilli in immune sera, prepared for each, and in practically identical end dilutions.

Other similar instances of cross-agglutinations are reported in the literature, but with few exceptions either the agglutination limit was less for the heterologous than for the homologous organism, or the coöperation of the two organisms in producing the agglutinins could not be excluded. Thus Stern (1898) studied five samples of blood serum from patients with typhoid fever which agglutinated, equally with the typhoid bacilli, the cultures of *B. coli* isolated from their stools. In this example, the possibility exists that mixed infection with the colon bacilli coexisted. Rodet (1897) found that sheep sera derived from animals immunized with *B. typhosus* and *B. coli*, respectively, agglutinated the heterologous organism equally with the homologous. Park and Williams (1910) observed the serum of a horse immunized with the Flexner dysenteric bacilli which agglutinated a culture of *B. coli* in the same end dilutions (1: 10,000) as the dysenteric bacillus. Conversely, a goat immunized with the *B. coli* culture yielded a serum of a titre of 1: 5,000 for the *B. coli* and 1: 3,000 for Flexner *B. dysenteriae*. The chief interest of the following communication arises from the fact that it concerns a similar cross-agglutination of *B. coli* and *B. dysenteriae* Shiga.

[1] Work conducted under a grant of the International Health Board of the Rockefeller Foundation.

EXPERIMENTAL

The culture of the *B. coli* used in the tests was recently obtained from the stool of a chronic carrier of *B. typhosus*. On inoculation into rabbits it proved pathogenic, but never induced the paralysis characteristic of the Shiga bacillus inoculation of that animal. The Shiga bacillus had been cultivated outside the body for a

TABLE 1

Particular and general agglutination tests with special B. coli and B. dysenteriæ sera

CULTURE		DYSENTERY SERUM						COLON SERUM					
		100	500	1000	2000	4000	C	100	500	1000	2000	4000	C
B. dysenteriæ (Shiga)	30	++	++	++	+	±	−	++	++	++	++	+	−
	6	++	−	−	−	−	−	+	−	−	−	−	−
	27	−	−	−	−	−	−	−	−	−	−	−	−
B. coli	13	++	++	++	+	±	±	++	++	++	++	+	±
	11	−	−	−	−	−	−	±	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	−	−	−	−
	14	−	−	−	−	−	−	−	−	−	−	−	−
	95	−	−	−	−	−	−	−	−	−	−	−	−
	104	−	−	−	−	−	−	−	−	−	−	−	−
	137	−	−	−	−	−	−	−	−	−	−	−	−
B. dysenteriæ (Flexner)	2	−	−	−	−	−	−	±	−	−	−	−	−
	22	−	−	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−	−	−	−	−	−
	25	−	−	−	−	−	−	−	−	−	−	−	−
	26	±	−	−	−	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−	−	−	−	−
	29	−	−	−	−	−	−	−	−	−	−	−	−
	S	−	−	−	−	−	−	−	−	−	−	−	−

long time; the original culture was obtained from Dr. Wadsworth of the New York State Department of Health. It fulfilled all the morphological and cultural requirements and upon inoculation into rabbits induced the typical paralysis. The two organisms had the property in common of tending to spontaneous agglutination.

The property of reciprocal agglutination was limited to the two strains described. Other cultures of *B. coli* and *B. dysenteriae* Shiga did not exhibit it. That is, agglutinating sera prepared with the particular strains of the colon and Shiga dysentery bacilli mentioned reacted on each other quite as on their own cultures, while other colon bacilli showed no especial agglutinating capacity with the Shiga immune serum nor did other Shiga bacilli, with the colon agglutinating serum. Similarly the stock poly-

TABLE 2

Absorption tests for agglutinins performed with homologous and heterologous cultures

SERUM	ABSORBING CULTURE	AGGLUTINATION TEST CULTURE	DILUTIONS						
			250	500	1000	2000	4000	8000	C
30	13	13	−	−	−	−	−	−	−
		30	++	++	+	±	±	−	−
	30	13	±	−	−	−	−	−	−
		30	+	−	−	−	−	−	−
13	13	13	±	−	−	−	−	−	−
		30	+	−	−	−	−	−	−
	30	13	++	++	++	++	++	−	−
		30	++	+	−	−	−	−	−
Controls.... 30	None	13	++	++	++	+	−	−	−
		30	++	++	++	++	−	−	−
13	None	13	++	++	++	++	++	−	−
		30	++	++	++	++	+	±	−

valent dysenteric horse serum agglutinated the particular *B. coli* strain at 1: 2000, which was its value to its homologous serum.

Single colonies of each organism were selected after several platings and employed to immunize rabbits. The injections were given at three day intervals. The rabbit receiving the culture of *B. coli* was given five intravenous injections, beginning with 1/100 and ending with 1/10 of an agar slant of living culture. The rabbit receiving the culture of the Shiga bacillus received seven intravenous injections, beginning with 1/50th

of a slant of a killed culture and ending with 1/10th of the living culture.

The results with the sera prepared from isolated colonies of each bacillus coincided with those of the original test. The fact should be mentioned that the particular Shiga immune serum was without agglutinating action on other Shiga cultures or cultures of the Flexner dysenteric group. Table I brings out in brief form the points just described. The cross-agglutinating colon bacillus culture is number 13 and the Shiga bacillus culture number 30.

Absorption of the immune sera was carried out with the homologous and heterologous agglutinating cultures, after which agglutination tests were made. The results are given briefly in table 2.

SUMMARY

Two cultures are described, one a typical *B. coli* and the other a typical *B. dysenteriae*, Shiga, culture, which yield immune sera possessing agglutinating properties of practical equal quantity for each culture. Absorption experiments made with each culture upon each kind of immune serum indicate that two distinct agglutinins are yielded in about equal amount in the process of immunization of rabbits with the respective cultures. The two agglutinins are specific ones, each for its own culture, and accessory (paragglutinin), each for the other culture. The absorption of the accessory agglutinin leaves the specific agglutinin quantitatively unaffected.

The significance of this reciprocal agglutinative property in respect to the two bacilli described can only be surmised. The obvious suggestion is a group relationship between certain strains of colon and dysenteric bacilli, a subject hardly to be pursued profitablv in this connection.

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COMMENTS ON THE EVOLUTION AND CLASSIFICATION OF BACTERIA

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The incompleteness of our present knowledge as to the species of bacteria and their relationships makes it very easy to criticise what others have written upon their evolution and to propose modifications in the classification of the group which in their turn are open to equal criticism. There has been so much speculation along this line recently[1] that a certain amount of confusion has resulted, and there is danger of increasing this confusion by discussing the subject from a new point of view. There would be little justification, indeed, for doing so, were it not for the fact that Jensen (1909) has already drawn up a detailed ancestral tree for the known groups of bacteria which has considerable merit and that the committee on classification appointed by the Society of American Bacteriologists (Winslow, et al., 1917b) has accepted many of Jensen's suggestions. This committee has given the matter so much thought and has drawn up such a well formulated report that there is danger of their recommendations being accepted *in toto* without sufficient scrutiny. Underlying their classification are certain assumptions as to the evolution and relationships of bacteria that should be thoroughly discussed before the report is adopted by the society.

In discussing the relationships of bacteria, it is necessary to keep in mind—just as when dealing with higher forms of life—that the living species represent only the ends of evolutionary lines, and that one modern form must not be considered the ancestor of another. It is probably true that there has been a greater persistence of primitive types among bacteria than in any other group of animals or plants, because the environment

[1] See Jensen (1909), Buchanan (1917) and Kligler (1917).

of many bacteria—salt water, fresh water and soil—has presented fairly uniform conditions throughout long geologic periods. Yet we can hardly assume that all of the primitive types of bacteria are still living. In fact, it would be more in harmony with what is known of the persistence of species and genera among higher forms of life—especially when the very brief life-cycle of bacteria is considered—to assume that the primordial types are all extinct. This idea seems to have been overlooked by Kligler in his recent paper (1917). Jensen in general kept the fact well in mind that he was dealing with end-products only; yet he also overlooked it at times, and failed to incorporate it into the diagram which he drew up to show the relationships of the different groups. In this diagram Jensen places the cephalotrichic organisms at the base of three main lines of development instead of representing them as a main branch coördinate with the peritrichic forms. Similarly Kligler makes no attempt to indicate that the Pseudomonas line of development appears to be as diverse as any other line; and has even indicated modern pathogenic forms (diphtheroids and albococci) as the ancestors of micrococci, a group which presumably includes the primitive cocci of soil.

This fallacious line of reasoning has led to certain questionable conclusions as to the relationships of bacteria, some of which have even been accepted by the committee on classification. The best way to point out this weakness in their report is to examine critically the different groups which they propose to establish.

The orders. The committee on classification recognizes four orders, Myxobacteriales, Thiobacteriales, Chlamydobacteriales, and Eubacteriales, together with an appendix, Spirochaetaceae. It is to be noted that they place Eubacteriales last, although it contains the simplest forms, while it is customary to arrange classifications in such a way that the simpler groups are placed at the beginning. The committee, indeed, has used the latter plan in arranging the classification of the Eubacteriales, as their first family, Nitrobacteriaceae, is the one they consider most primitive. In this respect there is an evident inconsistency

between the arrangement of their orders and the arrangement of their families.

Buchanan, in his recent classification (1917), recognizes six orders instead of four. He obtains this number by giving ordinal rank to the Spirochaetes and Actinomycetes. There is considerable reason to question whether the Spirochaetes belong with the Schizomycetes or with the Protozoa, so perhaps the committee's treatment of them is as satisfactory as Buchanan's. The Actinomycetes, however, differ so decidedly from true bacteria and embrace such a diversity of species[2] that there is good reason for accepting Buchanan's suggestion that there be a separate order Actinomycetales.

In passing, it is of interest to notice that there is a close analogy between the generally recognized groups of bacteria and those of Protozoa. Thus the cephalotrichic and peritrichic bacteria find their analogues respectively in the Flagellates and Ciliates (Infusoria). This analogy goes further than a mere resemblance in the arrangement of organs of locomotion; for the Ciliates and the peritrichic bacteria are both highly specialized groups, while both Flagellates and cephalotrichic bacteria contain all gradations between primitive forms and highly specialized human parasites. There is also a less striking analogy between the Rhizopods and the cocci, both groups with equal diameters. More striking is the resemblance between the two highly specialized, but apparently unrelated groups, the Myxobacteria and the Mycetozoa. Probably this analogy has no greater significance than the similar one so often mentioned between marsupials and placental mammals, in both of which groups the lines of development show much superficial similarity. Under similar conditions, different groups of living things frequently appear to undergo evolutionary development along parallel lines. This similarity between the groups of bacteria and of Protozoa does not indicate interrelationship; but it does increase the probability that the orders of bacteria just men-

[2] The diversity of species of Actinomycetes has been discussed by Lachner-Sandoval (1898), Neukirch (1903) and Conn (1917).

tioned represent the lines of development of the bacteria more truly than the physiological groups proposed by Jensen.

It is next necessary to take up in turn the seven families into which the committee has divided the order Eubacteriales.

Family 1. Nitrobacteriaceae. The justification for the recognition of this family is given in the following quotation from the committee report (p. 542):

> The Nitrobacteriaceae are clearly the most primitive of the Eubacteriales. Their power to live without complex organic substances would have made it possible, as Jensen points out, for them to flourish at a very early period in the world's history, and their simple structure is in harmony with the view that they represent the ancestral type of all other bacteria.

To accept this family, then, is really to endorse the theory that its members are modern representatives of the primordial bacteria.

Before endorsing that theory, however, it seems well to consider what evidence we have concerning the primordial types of bacteria. Such evidence cannot be obtained by paleontology, as when higher forms of life are concerned. What evidence we have from fossils as to the existence of bacteria in past ages, although scanty, is very interesting.[3] Recent deductions, however, such as those of Kligler (1917, p. 166) and of Osborn (1916, p. 292, and 1917, p. 86), as to the kinds of bacteria living in these early days, based upon the supposed protoplasmic structure of these fossilized organisms, will hardly be accepted by conservative bacteriologists.

Jensen's speculations as to the earliest forms of life are based upon chemical and physical considerations rather than upon paleontology and perhaps have a firmer foundation than those which rest upon Walcott's observations. Jensen concludes that the autotrophic bacteria were the first organisms on the earth, because they are the only known forms of life which can live upon inorganic matter without the action of sunlight, and

[3] See Walcott's evidence (1915) as to bacteria in Algonkian limestone, and Moodie's discussion (1916) of their existence in Mesozoic times.

he assumes that the primordial organisms must have lived in darkness and have depended upon inorganic matter for nutrition and energy. He overlooks the fact that if the earliest protoplasm possessed any such valuable property as the ability to utilize the chemical energy of inorganic material, it is strange that all but a very few of the organisms found today should have lost the power. He does not mention the idea that autotrophism may be a specialization developed by bacteria later on in the course of evolution; and yet the very organism which he selects as probably the most primitive of autotrophic bacteria, *B. methanicus* Söhngen (renamed *Methanomonas* by Jensen), derives its energy from methane liberated in swamps from decomposing organic matter. In other words, it gets its energy indirectly—through other organic agencies—from sunlight. Jensen's claim that under primordial conditions it utilized methane produced by volcanic action is a rather improbable assumption. It might be possible to advance more valid arguments in favor of the greater primitiveness of the nitrifying bacteria. Jensen does not deny this possibility; but he plainly believes that the most primitive organism is to be found among the autotrophic bacteria.

The committee on classification has apparently accepted this argument of Jensen's, as seen by a glance at the genera composing the family Nitrobacteriaceae. The first seven genera of Nitrobacteriaceae listed by the committee are the same and are even arranged in the same order as the seven genera placed by Jensen in his most primitive family, the Oxydobacteriaceae, except that for three of the genera the committee has recognized the validity of names prior to those of Jensen. It is evident that if the society adopts the committee report, it will be committing itself as favoring Jensen's arguments, and before doing this it is necessary to be sure that there are no other equally tenable theories as to primordial life.

As a matter of fact, other theories have been advanced which are by no means disproved by Jensen's arguments. Most important of them is the one that looks to the blue-green algae or to the little known phototrophic pigment-containing bacteria as the primordial organisms. These organisms, like

the autotrophic bacteria, use inorganic food, but they obtain their energy from sunlight instead of from chemical transformations. Jensen disregards this theory on the assumption that the primordial earth was dark; but Chamberlin's theory of the earth's origin has as much weight today as the nebular hypothesis, and according to his theory sunlight may have reached the earth's surface even in the earliest time.[4]

Another possibility not to be overlooked is that the earliest bacteria had energy-bearing carbon compounds at their disposal. These may have been formed by preceding life of still simpler nature, which is either extinct today or has escaped detection; or they may have been formed by inorganic agencies. Moore and Webster (1913) have shown that organic matter (formaldehyde) can be synthetized by the action of certain inorganic catalysts, which utilize sunlight energy, and have pointed out the significance of this fact as a possible explanation of the source of nutrition for the earliest life on the earth. If we assume that organic matter was synthesized by inorganic agencies before the existence of life, it is entirely unnecessary to look to phototrophic or autotrophic organisms as the original ancestors. The first organisms might have derived both food and energy from the simple carbon compounds then in existence.

The only justification for recognizing several genera of autotrophic bacteria is on the assumption that the few species we know are the sole survivors of primitive genera. This assumption, as just shown, is not the only reasonable hypothesis. A more conservative course than to accept the entire list of genera of Nitrobacteriaceae would be to recognize but one, or at the most, two genera of prototrophic bacteria. If two genera are to be recognized, one could include those forms capable of obtaining both their carbon and nitrogen from inorganic sources, and the other those requiring organic carbon but able to use elementary nitrogen. For these two genera, the names Nitrosomonas Winogradsky and Azotobacter Beijerinck would have to stand by priority.

[4] See Chamberlin (1916), p. 248. An earlier discussion of primordial conditions by Chamberlin and Chamberlin (1908) shows that life may perhaps have started under conditions not so very different from those of today.

There is no reason, indeed, for making a separate family of the phototrophic bacteria. As Jensen points out, most of them are rods with polar flagella, a fact which suggests that they are closely related to the heterotrophic *Pseudomonas* forms. They may easily be placed in the family Pseudomonadaceae recognized by the committee. It is true, as remarked by Buchanan (1917), that the spherical *Nitrosococcus* should not be separated from the rod-shaped *Nitrosomonas;* but this can easily be avoided without making a separate family for these organisms. One of Jensen's most valuable contributions to systematic bacteriology is in pointing out that the shape of cell or form of body is not a fundamental character and that there is not only a possibility but even a probability that transformations of cocci to rods and of rods to cocci may have taken place more than once in the course of development. This conception makes it entirely possible to place the autotrophic and possibly some simple heterotrophic cocci in the Pseudomonadaceae. It would merely require a definition of the family so worded as not to exclude all coccus forms.

The acceptance of Jensen's ideas as to the transformations of cocci into rods and rods into cocci does not force us to accept his theory that the most primitive forms were rods with polar flagella. In the natural course of development, a non-motile spherical organism would probably have preceded the motile, rod-shaped *Pseudomonas* type. As soon as a spherical organism becomes motile it tends to become elongated, a fact which undoubtedly explains the rarity of motile cocci. This thought gives another reason for hesitation before accepting the family Nitrobacteriaceae as the most primitive group of bacteria.

Family 2. Mycobacteriaceae. This family is placed by the committee immediately after the Nitrobacteriaceae. In this they follow Jensen. Jensen, however, uses this arrangement on the fallacious assumption that *Rhizobium* (the organism of legume nodules) has a polar flagellum, as formerly claimed by Harrison and Barlow (1907). On this assumption *Azotobacter* and *Rhizobium,* both with polar flagella and both able to utilize atmospheric nitrogen, formed natural stepping-stones between

the two groups. Jensen placed *Azotobacter* in one family and *Rhizobium* in the other. In their report as originally presented (Winslow, et al., 1917a), the committee on classification did the same; but in their complete published report (1917b) they have placed both *Azotobacter* and *Rhizobium* in the Nitrobacteriaceae.

This change may have been unintentional; in which case it is a very unfortunate mistake. DeRossi (1906), Zipfel (1911) and Kellerman (1912) and others have shown conclusively that the proper technic reveals peritrichous flagella on the legume organism.[5] The characterization of the genus given by the committee on page 553 of their report is therefore incorrect. That peritrichic organisms with granular structure and branching cells should be placed in the same family with the autotrophic bacteria can be justified only if physiology is allowed to override morphology entirely in establishing the classification. The committee distinctly state in regard to the Nitrobacteriaceae (p. 551) "When motile, with polar, never peritrichous, flagella."

The treatment of *Rhizobium* is important, because its relationships probably determine the position of the *Actinomyces* line. On the one hand its branching cells and granular structure show a striking resemblance to the tubercle organism and Actinomycetes, while on the other hand the motility of its vegetative rods helps to establish a connection with the true bacteria. Jensen accepted the old description of *Rhizobium* as a monotrichic rod and therefore placed the family of Actinomycetes in the Cephalotrichinae; but as the legume organism has now been definitely

[5] After finishing this paper the writers have noticed that Burrill and Hansen (Ill. Agr. Exp. Sta., Bul. 202. 1917) have apparently observed a single flagellum on this organism. As stated, however, by Hansen (who wrote the bulletin), this flagellum seems to be at a corner instead of at the pole, and the figures show it at times attached to the middle of the rod. This strongly suggests preparations that the writers have seen of known peritrichic organisms which, because of poor technic, reveal only a single flagellum on a rod. The work of such careful investigators as DeRossi, Kellerman, Zipfel and Prucha ought not to be dismissed with the few words granted to them by Hansen, while the still more recent work of Wilson (Cornell Agr. Exp. Sta., Bul. 386. 1917) apparently has not been seen by Hansen. The writers have seen microscopic preparations made by Kellerman which are very much better than his published photomicrographs and show peritrichous flagella without any question.

shown to have peritrichous flagella, the family is probably derived from the peritrichic stem, and does not belong near the Nitrobacteriaceae, where it has been placed by the committee. Buchanan, as previously mentioned, has wisely suggested the recognition of a separate order, calling it Actinomycetales. Perhaps the order might be subdivided into two families: Mycobacteriaceae for forms like the tubercle organism, Actinomycetaceae for the forms with true branched filaments.

All the genera in this group recognized by the committee probably have good standing, with the possible exception of *Nocardia* which as defined has no suitable type species.

Family 3. Pseudomonadaceae. This family has good standing, as does the genus *Pseudomonas*, also; but as already mentioned, the family might include the autotrophic as well as the heterotrophic forms. The definition of the family should be so worded as not to exclude all spherical or immotile forms that seem to be closely related to typical species. The committee has evidently made a mistake in saying "Flagella *single*, polar," thus leaving no place for the lophotrichic rods. Many of the fluorescent water bacteria which the committee plainly intends to put in this family have a tuft of four or more polar flagella.

Family 4. Spirillaceae. The arrangement of this family in two genera, as recognized by the committee, is apparently as satisfactory as any which can be suggested.

Family 5. Coccaceae. This family has been studied so thoroughly by Winslow and Winslow (1908) and its nomenclature given such careful scrutiny by Buchanan (1915) that there are no good reasons for criticising its genera as recognized by the committee.

The question arises whether this family is primitive or highly specialized. On the one hand the spherical form suggests primitiveness, while on the other the large number of animal parasites it contains plainly indicates recent development. As a matter of fact, the group seems to contain all gradations from soil and water forms that live on material of fairly simple composition up to parasites that are adapted to growth in living animals. Perhaps eventually we will have to accept Jensen's

theory that the spherical bacteria do not constitute a distinct evolutionary group but that the parasitic cocci are of an independent origin, more modern than the saprophytic ones.

The position of the genus *Streptococcus* is a puzzling matter. Jensen holds that rods gave rise to streptococci and that micrococci were derived from streptococci. This argument can be used only on the assumption just mentioned that the saprophytic cocci had an earlier independent origin from some other source. There are good reasons, moreover, for thinking that streptococci are more closely related to the chain-forming short rods occurring in milk than to the micrococci; while some bacteriologists place them near the diphtheroids (Mellon, 1917). Kligler (1917) separates them widely from the micrococci. The streptococci, in short, might easily be removed entirely from the Coccaceae; but because of the uncertainty of the whole matter, it seems best to be conservative and leave them, as the committee recommends in the same family with the other spherical bacteria.

Family 6. Bacteriaceae. There is some question just what this family should include. The committeee makes it include practically all peritrichic and non-motile, non-spore-forming rods, exclusive of the Bulgaricus group. The chief objection to this arrangement is that it tends to place in this family any member of the Pseudomonadaceae that has lost its power of motility. A non-motile, non-spore-forming rod cannot safely be assigned to either of these families until it has been studied sufficiently to make plain its relationships to other bacteria. A possible treatment of the non-motile, non-spore-forming rod-shaped bacteria would be to recognize an appendix to the families of Eubacteriales in which they may be placed until their relation to other better defined species is learned. The mycologists recognize a group which they call Fungi Imperfecti. Bacteriologists might equally well create a group of Bacteria Imperfecta.

The committee has placed four genera in the Bacteriaceae. The chief criticism against the selection of these genera is that too great weight has been placed upon pathogenicity. Two of them, *Hemophilus* and *Pasteurella*, include animal pathogens only; one, *Erwinia*, includes only plant pathogens; while the

third, into which apparently are to be dumped all peritrichic or non-motile, non-spore-forming saprophytes, is stated in the report to consist primarily of the colon-typhoid-dysentery group. It is particularly unfortunate that *B. coli* has been proposed as the type of this genus, since the group which centers around this organism is quite distinct from the majority of saprophytes belonging in this family. In this matter they claim to be accepting Jensen's emendation of the genus *Bacterium;* but they expand his emendation to include his *Denitrobacterium* and also the *Proteus* group, which Jensen named *Liquidobacterium*, and have actually, therefore, proposed a new emendation. If *B. coli* is accepted as the type of *Bacterium* it would be natural to use the generic name only for the colon-typhoid group, leaving no place for the large number of indefinite forms that the committee intends it to include.

Still more questionable is the genus *Erwinia*. This has been named as a new genus by the committee without a type species and therefore does not have a good standing. It is very doubtful, indeed, whether peritrichic plant parasites are sufficiently distinct from saprophytes to be put in a genus by themselves.

It seems strange that the committee has not recognized the genus *Proteus* Hauser, which is as distinct as several that they have recognized. Although this genus is primarily saprophytic, it has a fairly well defined type species, and is apparently distinct from the colon-typhoid group.

Family 7. Lactobacillaceae. There is very little reason for putting the Bulgaricus type of organisms in a family by themselves. They do differ in many respects from the Gram-negative rods—enough to justify the recognition of the genus *Lactobacillus*, but hardly enough to furnish a basis for the establishment of a new family. The very points of greatest distinction, granular structure, occurrence of bud-like branches, and failure to decolorize by the Gram method, are points which suggest a relationship to the diphtheria and tubercle organisms. So little is known about the *Lactobacillus* group, however, that there is scant justification for placing it in the Mycobacteriaceae, and the

most conservative procedure is to leave these forms in the Bacteriaceae.

Family 8. Bacillaceae. This family, for the spore-forming rods, has very good justification. The two genera, *Bacillus* and *Clostridium*, can probably be separated, but whether on the basis of relation to oxygen or of shape of the sporangium, the future must decide. Although relation to oxygen is a very important physiological distinction, it must be admitted that the selection of a physiological basis for the separation of these two genera is rather unsatisfactory. It places some of the polar-spored organisms in one genus, some in the other, and raises the question where to place facultative anaerobes like *B. mycoides* and *B. cereus*.

Type species. At the 1916 meeting of the society, the committee on classification proposed that American bacteriologists follow the international code of nomenclature adopted at the Vienna Botanical Congress. No opportunity had been given the society to study into the matter, and naturally the resolution was passed. It seems, however that the Vienna code does not recognize the principle of type species. The committee on classification (p. 531, footnote) states that the principle of type species "affects bacterial taxonomy less than other divisions of taxonomy." In this opinion they are decidedly open to criticism; for the very difficulty in defining bacterial genera and species accurately makes it imperative that no genus be recognized without a well-defined type. The fact that the society took action in favor of the Vienna code does not absolve bacteriologists from accepting this fundamental principle of nomenclature as it is recognized by zoölogists and by many botanists.

The committee has plainly considered type species unnecessary, for they have omitted to mention them for the following genera: *Nocardia*, *Pseudomonas*, *Spirillum*, *Albococcus*, *Rhodococcus*, and *Erwinia*. Some of these genera may easily be assigned type species (e.g., *Albococcus pyogenes* (Rosenbach) Winslow); but for others, such as *Nocardia*, none is available. It is especially unfortunate that the committee has neglected to name a type species for *Erwinia*, which they describe as a new genus.

CONCLUSIONS

In spite of all these criticisms of the report in matters of detail, it should be recognized that the committee has drawn up an admirable summary of the present status of systematic bacteriology. In matters of opinion, the names of the six members of the committee carry a great deal of weight. Even in matters of detail their classification is as satisfactory as could be drawn up by any other similar group of men. The report is, in reality, open to criticism mainly because of the extent of the undertaking which the society has placed upon the committee. No other committee on systematic biology appointed by a national or international society has ever undertaken such an ambitious task as a complete classification of any group of animals or plants. Other committees of this sort have done nothing further than to pass upon the validity of generic and specific names submitted to them, leaving it to individual initiative to propose new names, to classify and to define the groups.

Summing up the suggestions made above, the following classification of orders and families of Schizomycetes is obtained:

Order 1. Eubacteriales. Cells minute; spherical, rod-shaped, or spiral, often occurring in chains, but never in branched or sheathed filaments.

Family 1. Pseudomonadaceae. Cells generally rod-shaped, though occasionally spherical. If motile, flagella occur at the pole. No endospores. Generally Gram-negative. Primarily water and soil forms. Sometimes able to assimilate inorganic nitrogen and to obtain energy from the oxidation of inorganic compounds. Often parasitic to plants, seldom to animals.

Family 2. Spirillaceae. Cells more or less spirally curved. If motile, flagella are polar.

Family 3. Coccaceae. Cells in their free conditions spherical; during division somewhat eliptical. Motility rare. Endospores absent. Often parasitic on animals.

Family 4. Bacteriaceae. Rod-shaped cells without endospores. Generally Gram-negative. Flagella when present peritrichic. Saprophytes, animal parasites and plant parasites.

Family 5. Bacillaceae. Rods producing endospores, usually Gram-positive. Flagella, when present, peritrichous. Primarily saprophytes secreting proteolytic enzymes. A few parasites.

Appendix. Bacteria Imperfecta. A temporary group to contain the non-motile, non-spore-forming rods, whose relationship to any of the above families cannot be shown.

Order II. Actinomycetales. Cells usually elongated, frequently filamentous and with a tendency to the development of branches.

Family 1. Mycobacteriaceae. Cells do not form true branched filaments. Frequently show swellings, clubbed or irregular shapes. Gram-positive. Frequently pathogenic. Aerobic.

Family 2. Actinomycetaceae. True filaments formed, developing into a definite branched mycelium. One-celled reproductive bodies formed by fragmentation. Aerobic and anaerobic. Occasionally pathogenic, but ordinarily living upon simple organic compounds.

Order III. Thiobacteriales. Cells free or united in elongated filaments. Cells typically containing either granules of free sulphur or bacterio-purpin or both, usually growing best in presence of hydrogen sulphide.

Order IV. Chlamydobacteriales. Cells normally in elongated filaments. Iron often present. Usually a well-marked sheath.

Order V. Myxobacteriales. Cells united during the vegetative state into a pseudoplasmodium which passes over into a highly developed cyst-producing resting stage.

Appendix. Spirochaetaceae. Spirilliform organisms, multiplying by transverse division. Frequently parasitic.

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STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

IX. THE SUBGROUPS AND GENERA OF THE THIOBACTERIALES

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Order IV. **Thiobacteriales** Ordo nov.

Cells various, typically containing either granules of free sulphur, or bacteriopurpurin, or both, usually growing best in the presence of hydrogen sulphide. The cells are plant-like, not protozoan-like, not producing a pseudoplasmodium or a highly developed encysted resting stage. Spores are rarely or never formed.

Classification within this group is in a very unsatisfactory and very superficial state. Few investigators have studied these forms, and most of the work is old, and in need of careful revision. Undoubtedly many of the genera are to be regarded as growth forms, merely.

The following names have been applied to families, subfamilies, tribes and subtribes.

Chromatiaceae Migula, 1900, p. 1047
Amoebobacterieae De Toni and Trevisan, 1889, p. 1043
Beggiatoaceae Migula, 1895, p. 41
Amoebobacteriaceae Migula, 1900, p. 1045
Thiocapsaceae Migula, 1900, p. 1042
Thiopediaceae Migula, 1900, p. 1044
Rhodobacteriaceae Migula, 1900, p. 1042
Lamprocystaceae Migula, 1900, p. 1043
Athiorhodaceae Molisch, 1907, p. 28
Thiobacteriaceae Jensen, 1909, p. 303

The order Thiobacteriales may be divided into families as follows:

Key to the families of Thiobacteriales

A. Cells containing sulphur granules (or in one species possibly oxalate crystals), but no bacteriopurpurin.
  1. Unicellular, motile forms. Not filamentous. Family I. *Achromatiaceæ*
  2. Filamentous forms ..... Family II. *Beggiatoaceae*
B. Cells containing bacteriopurpurin with or without sulphur granules. Family III. *Rhodobacteriaceæ*

Family I. **Achromatiaceae** Fam. nov.

Unicellular, large, motile (by means of flagella?) cells containing granules of sulphur (or in one form possibly oxalate), but no bacteriopurpurin.

The following key will separate the genera recognized.

Key to the genera of Achromatiaceae

A. Cells spherical or ellipsoidal
  1. Cells ellipsoidal (spherical when newly divided). Cells containing granules of calcium oxalate (perhaps sulfur). Genus I. *Achromatium*
  2. Cells spherical, with sulphur granules in a central vacuole. Genus 2. *Thiophysa*
B. Cells longer, very large (42 to 86μ) with peritrichous flagella. Genus. 3. *Hillhousia*

Genus 1. **Achromatium** Schewiakoff 1893

Synonyms:

Modderula Frenzel, 1897, p. 901

Cells large, nearly spherical in newly divided cells to ellipsoidal, 15 to 43 by 9 to 22μ. Cells closely packed with large granules, at first interpreted as sulphur, but later interpreted as calcium oxalate. When granules are dissolved, cells show coarse alveolar structure. Cells are motile, flagella not demonstrated. Cell division resembles the constriction of flagellates rather than the fission characteristic of bacteria.

The type species is *Achromatium oxaliferum* Schewiakoff. The organism occurs in the slime at the bottom of the rivers, in the so called "Modder."

Genus 2. **Thiophysa** Hinze, 1903, p. 310

Spherical cells laden with sulphur. The protoplasmic layer surrounds a large central vacuole. Cell nucleus not recognized. Flagella lacking. Cells elongate before division, divide to biscuit shaped cells. Cells 7 to 18μ in diameter.

The type species *Thiophysa volutans* Hinze was secured from the Bay of Naples.

Genus 3. **Hillhousia** West and Griffiths, 1909, p. 398

Cells very large, 42 to 86 by 20 to 33μ. motile by means of peritrichous flagella. Cells packed with large globules of oily amorphous sulphur.

The type species is *Hillhousia mirabilis* West and Griffiths.

Family II. **Beggiatoaceae** Migula, 1895, p. 41

Filamentous bacteria, usually showing an oscillating motion similar to Oscillatoria. Cells contain sulphur granules. Spore formation and conidia unknown.

The genera of the family *Beggiatoaceae* may be differentiated by means of the following key:

Key to the genera of Beggiatoaceae

A. Filament non-motile, with a contrast to base and tip, attached.
Genus 1. *Thiothrix*

B. Filaments motile (oscillating) not attached, no differentiation into tip and base.
1. Filaments not in bundles nor surrounded by a gelatinous sheath.
Genus 2. *Beggiatoa*
2. Filaments in bundles, surrounded by a gelatinous sheath.
Genus. 3. *Thioploca*

Genus 1. **Thiothrix** Winogradsky, 1888, p. 39

Filament non-motile, segmented, a definite differentiation into base and tip, attached, usually filled with sulphur granules. The threads produce rod shaped conidia at their ends. These conidia are self motile by means of a slow creeping motion, attach themselves and develop into new threads. The habitat is hot sulphur springs.

The type species is *Thiothrix nivea.* (Rabenhorst) Winogradsky.

Genus 2. **Beggiatoa** Trevisan, 1842, p. 76

Threads sheathless, formed of flat discoidal cells, not attached. Multiplication by transverse splitting of the threads. Showing an undulating motion, creeping. Cells contain globules of sulphur. Usually in hot sulphur springs.

The type species is *Beggiatoa alba* (Vaucher) Trevisan.

Genus 3. **Thioploca** Lauterborn, 1907, p. 238

Filaments Beggiatoa-like, with numerous sulphur granules, motile, lying parallel in considerable numbers, or united in bundles enclosed in a colorless layer of gelatin.

The type species, *Thioploca schmidlei* Lauterborn has filaments 5 to 9μ thick, and gelatinous sheath 50 to 160μ thick. From the ocean bed.

Family III. **Rhodobacteriaceae** Migula, 1900, p. 1042

Synonym:

Rhodobacteria Molisch, 1907, p. 27

Cells of various types, not filamentous, containing bacterio-purpurin with or without sulphur granules also.

Two subfamilies may be separated by the following key:

Key to the subfamilies of Rhodobacteriaceae

A. Cells containing sulphur granules.....Subfamily I. *Chromatioideae*
B. Cells without sulphur granules.....Subfamily II. *Rhodobacterioideae*

Subfamily I. **Chromatioideae** Nom. nov.

Synonyms:

Thiorhodaceae Molisch, 1907, p. 28

Cells not filamentous, containing both sulphur granules and bacteriopurpurin.

The following names have been used for genera in this group:

Erythroconis Oersted, 1842, p. 552
Chromatium Perty, 1852, p. 179
Clathrocystis Henfrey, 1856, p. 53
Rhabdomonas, Cohn, 1875, p. 167

Cohnia Winter, 1884, p. 48
Lamprocystis Schroeter, 1886, p. 151
Lampropedia Schroeter, 1886, p. 151
Mycothece Hansgirg, 1888, p. 266
Amoebobacter Winogradsky, 1888, p. 71
Thiocapsa Winogradsky, 1888, p. 84
Thiocystis Winogradsky, 1888, p. 60
Thiodictyon Winogradsky, 1888, p. 80
Thiopedia Winogradsky, 1888, p. 85
Thiopolycoccus Winogradsky, 1888, p. 79
Thiosarcina Winogradsky, 1888, p. 104
Thiospirillum Winogradsky, 1888, p. 104
Thiothece Winogradsky, 1888, p. 82
Cenomesia De Toni and Trevisan, 1889, p. 1039
Thiosphaerion Miyoshi, 1897, p. 170
Thiosphaera Miyoshi, 1897, p. 170
Rhodocapsa Molisch, 1906, p. 223
Rhodothece Molisch, 1906, p. 223
Amoebomonas Jensen, 1909, p. 338
Thioderma Miyoshi, 1897, p. 170
Rhabdochromatium Winogradsky, 1888, p. 100

Of these names *Clathrocystis* and *Erythroconis* are algal genera to which certain of the sulphur bacteria have at different times been assigned.

Following is a key to the tribes of the *Chromatioideae* which may be recognized largely from the descriptions of Winogradsky.

Key to the tribes of Chromatioideae

A. Cells united, at least during a part of the life history, into families.
  I. Cell division such that masses of cells, not merely plates, are formed.
    a. Cell division in three directions of space.....Tribe I. *Thiocapseae*
    b. Cell division first in three, then in two directions of space.
      Tribe II. *Lamprocysteae*
  II. Cell division in two planes, forming plates of cells.
      Tribe III. *Thiopedieae*
  III. Cell division in one plane.....Tribe IV. *Amoebobacterieae*
B. Cells free, capable of swarming at any time.....Tribe V. *Chromatieae*

Tribe I. **Thiocapseae** Trib. nov.

Synonyms:

Thiocapsaceae Migula, 1900, p. 1042

Bacteria containing both sulphur granules and bacteriopurpurin. Cells divide in three directions of space, united into families.

Key to the genera of the Thiocapseae

A. Cells capable of swarming.
  I. Families small, compact, enclosed singly or several together in a cyst.....Genus I. *Thiocystis*
  II. Cells large, 7 to 8μ loosely bound by gelatin into loose families Genus II. *Thiosphaera*
  III. Cells small, united into solid, spherical families. Genus III. *Thiosphaerion*

B. Cells not capable of swarming.
  I. Spherical cells spread out upon the substratum in flat families, loosely enveloped in a common gelatin.....Genus IV. *Thiocapsa*
  II. Arranged in regular packets like *Sarcina*.....Genus V. *Thiosarcina*

Genus I. **Thiocystis** Winogradsky, 1888, p. 60

Usually 4 to 20 or 30 cells massed into small, compact families, enveloped singly or several together in a gelatinous cyst, capable of swarming. When the families have reached a definite size they escape from the gelatinous cyst, the latter either swelling and softening uniformly or at some particular spot. The escaped cells either pass into the swarm stage or unite into a larger fused complex of families. Cells are light colored, single cells almost colorless. In masses the cells show a beautiful violet or red violet color. The cells are frequently quite filled with sulphur granules.

The type species is *Thiocystis violacea* Winogradsky.

Genus II. **Thiosphaera** Miyoshi, 1897, p. 170

Cells spherical-ellipsoidal, relatively large (7 to 8μ) light violet in color, bound into loose families by a colorless gelatin. Capable of swarming. Sulphur inclusions relatively abundant.

The type species is *Thiosphaera gelatinosa* Miyoshi.

Genus III. **Thiosphaerion** Miyoshi, 1897, p. 170

Cells spherical-ellipsoidal, small (1.8 to 2.5μ violet in color, with delicate sulphur inclusions. United by means of gelatin into solid spherical families. Capable of swarming.

The type species is *Thiosphaerion violaceum* Miyoshi.

Genus IV. **Thiocapsa** Winogradsky, 1888, p. 84

Cell families resembling in grouping and multiplication the cells of the algal genus Aphanocapsa. Cell division occurs in all directions of space, the cells are spherical, with thick confluent membranes, which unite to form a structureless, gelatinous layer. The cells are of a bright rose red color and contain numerous sulphur granules. The cells do not swarm.

The type species is *Thiocapsa roseo-persicina* Winogradsky.

Genus V. **Thiosarcina** Winogradsky, 1888, p. 104

Synonym:

Rhodosarcina Jensen, 1909, p. 334

Non-swarming cells arranged in packet shaped families, corresponding to the genus Sarcina. Cells red, with sulphur granules.

The type species is *Thiosarcina rosea* (Schroeter) Winogradsky.

Tribe II. **Lamprocysteae** Trib. nov.

Synonyms:

Lamprocystaceae Migula, 1900, p. 1043

Cells united into families in which division of the cells occur first in three planes, then in two.

The single genus of this tribe is *Lamprocystis.*

Genus I. **Lamprocystis** Schroeter, 1886, p. 151

Synonyms:

Clathrocystis Cohn, 1875
not *Clathrocystis* Henfrey, 1856, p. 53
Cohnia Winter, 1884, p. 48
not *Cohnia* Kunth 1850
not *Cohnia* Reichenbach 1852
Cenomesia? De Toni and Trevisan, 1889, p. 1039

Cells ellipsoidal, dividing at first in three planes to form spherical cell masses, later in two planes, forming hollow sacks in which the cells lie embedded in a layer in the walls, finally the membrane ruptures, and the whole mass becomes net like, much as in the algal genus Clathrocystis. Usually colored intensely violet. Small sulphur granules present. Capable of swarming.

The type species is *Lamprocystis roseo-persicina* (Cohn) Schroeter.

Tribe III. **Thiopedieae** Trib. nov.

Synonyms:

Thiopediaceae Migula, 1900, p. 1044

Sulphur bacteria in which the cells are united into families, and cell division is in two directions of space, resulting in the development of plates of cells.

The two genera may be differentiated by the following key:

Key to the genera of Thiopedieae

A. Cells occurring regularly in fours.....Genus I. *Lampropedia*
B. Cells occurring in a film or membrane, not regularly disposed in tetrads. Genus II. *Thioderma*

Genus I. **Lampropedia** Schroeter, 1886, p. 151

Synonyms:

Erythroconis? Oersted, 1842, p. 6
Thiopedia Winogradsky, 1888, p. 85

Cells united into tetrads, forming flat tubular masses. Contain sulphur granules and bacteriopurpurin.

The type species is *Lampropedia hyalina* (Kuetzing) Schroeter.

Genus II. **Thioderma** Miyoshi, 1897, p. 170

Cells spheroidal, light rose in color, containing small, inconspicuous, sulphur granules. United into thin purplish membrane,

The type species is *Thioderma roseum* Miyoshi.

Tribe IV. **Amoebobacterieae** Trib. nov.

Synonyms:

Amoebobacteriaceae Migula, 1900, p. 1045

Sulphur bacteria in which the cells are united into families. Cell division occurring only in one direction of space.

Key to the genera of Amoebobacterieae

I. Cells connected by plasma threads, families amoeboid motile.
Genus I. *Amoebobacter*

II. Cells not as I.
A. Cells arranged in a net, united by their ends.....Genus II. *Thiodictyon*
B. Cells not arranged in a net.
1. Capable of swarming. Cells loosely aggregated in gelatin.
Genus III. *Thiothece*
2. Non-motile. Cells closely appressed into a colony.
Genus IV. *Thiopolycoccus*

Genus I. **Amoebobacter** Winogradsky, 1888, p. 71

Cells connected by plasma threads. Families amoeboid motile. The cell families slowly change form, the cells drawing together into a heap or spreading out widely, thus bringing about a change in the shape of the whole family. In a resting condition a common gelatin is extruded, the surface becomes a firm membrane.

The type species is *Amoebobacter roseus* Winogradsky.

Genus II. **Thiodictyon** Winogradsky, 1888, p. 80

Synonym:

Rhododictyon Jensen, 1909, p. 334

Cells rod-shaped or spindle-shaped, with sharply pointed ends, united into a net. The compact mass of rods finally assumes an appearance like that of Hydrodictyon. Slight violet color.

The type species is *Thiodictyon elegans* Winogradsky.

Genus III. **Thiothece** Winogradsky, 1888, p. 82

Cells spherical, in families enclosed in a thick gelatinous cyst. Cells capable of swarming and very loosely embedded in a common gelatin. When the swarm stage supervenes, the cells lie more

loosely, the gelatin is swollen, and the cells swarm out singly and rather irregularly.

The type species is *Thiothece gelatinosa* Winogradsky.

Genus IV. **Thiopolycoccus** Winogradsky, 1888, p. 79

Families solid, non-motile, consisting of small cells closely appressed. Multiplication of the colonies by the breaking up of the surface into numerous short shreds and lobes which continue to split up into smaller heaps. Cells red.

The type species is *Thiopolycoccus ruber* Winogradsky.

Tribe V. **Chromatieae** Trib. nov.

Synonym:

Chromatiaceæ Migula, 1900, p. 1047

Sulphur bacteria in which the cells are not united into families, but free, and capable of swarming at any time.

The genera of the tribe *Chromatieae* may be differentiated by the following key:

Key to the genera of Chromatieae

A. Cells motile by means of polar flagella. Elongated.
  I. Cells not spiral.
    a. Cells cylindric ..... Genus I. *Chromatium*
    b. Cells with tendency to spindle shape ..... Genus II. *Rhabdomonas*
  II. Cells spiral ..... Genus III. *Thiospirillum*
B. Cells spherical, or little elongate, non motile.
  I. Cells not encapsulated ..... Genus IV. *Rhodocapsa*
  II. Cells encapsulated in pairs ..... Genus V. *Rhodothece*

Genus I. **Chromatium** Perty, 1852

Synonym:

Rhodomonas Jensen, 1909, p. 334

Cells cylindric-elliptical or relatively thick cylindrical. Cell contents red, containing dark sulphur granules. Cells somewhat variable in shape, straight, more or less bent, short cells ovoid and longer forms more cylindrical. Motile by means of polar flagella.

The type species is *Chromatium okenii* Perty.

Genus II. **Rhabdomonas** Cohn, 1875, p. 167

Synonyms:

Mantegazzaea Trevisan, 1879, p. 137 in part
Rhabdochromatium Winogradsky, 1888, p. 150

Differentiated from Chromatium by the elongated rod-shaped or spindle-shaped cells. Cells red, with sulphur granules, polar flagella.

The type species is *Rhabdomonas rosea* Cohn.

Genus III. **Thiospirillum** Winogradsky, 1888, p. 104

Synonym:

Ophidomonas? Ehrenberg.

Spiral motile bacteria containing sulphur granules and bacteriopurpurin.

The type species is *Thiospirillum sanguineum* (Ehrenberg) Winogradsky.

Genus IV. **Rhodocapsa** Molisch, 1906, p. 223

Cells spherical, free (not united into families) not capable of swarming (non-motile). In mass the organisms are cherry red. Contain sulphur granules.

The type species is *Rhodocapsa suspensa* Molisch.

Genus V. **Rhodothece** Molisch, 1906, p. 223

Cells usually spherical and in pairs, each surrounded by a spherical or an ellipsoidal capsule. Non-motile. Cells not united into families. Cells contain bacteriopurpurin and sulphur granules.

The type species is *Rhodothece pendens* Molisch.

Subfamily II. **Rhodobacterioideae** Subfam. nov.

Synonym:

Athiorhodaceae Molisch, 1907, p. 28

Cells not filamentous, containing bacteriopurpurin but not granules of sulphur.

The genera of this subfamily have all been described by Molisch. They may be differentiated by the following key:

Key to the genera of Rhodobacteroideae

I. Cells united into families.
  A. Cells rod shaped, many embedded in the same slimy capsule. Genus I. *Rhodocystis*
  B. Cells spherical or short rods.
    1. In chains, each chain surrounded by a capsule. Genus II. *Rhodonostoc*
    2. Cells free..... Genus III. *Rhodosphaera*
  C. Cells free and elongate.
    1. Cells not bent.
      a. Non-motile..... Genus IV. *Rhodobacterium*
      b. Motile..... Genus V. *Rhodobacillus*
    2. Cells bent or curved.
      a. Cells short, comma shaped, with single polar flagellum Genus VI. *Rhodovibrio*
      b. Cells spiral, with polar flagella.. Genus VII. *Rhodospirillum*

Genus I. **Rhodocystis** Molisch, 1907, p. 22

Cells rod-shaped, dividing in only one plane embedded in a common slimy capsule.

The type species is *Rhodocystis gelatinosa* Molisch.

Genus II. **Rhodonostoc** Molisch, 1907, p. 23

Cells spherical or short rods, in rosary like chains, and embedded in a common gelatinous capsule.

The type species is *Rhodonostoc capsulatus* Molisch

Genus III. **Rhodosphaera** gen. nov.

Synonym:

Rhodococcus Molisch, 1907, p. 20
not *Rhodococcus* Zopf, 1891, p. 28

Cells spherical, non-motile, free not united into families.

The type species is *Rhodosphaera capsulatus* (Molisch) Buchanan.

Genus IV. **Rhodobacterium** Molisch, 1907, p. 16

Rod shaped cells, non-motile, not united into families.

The type species is *Rhodobacterium capsulatum* Molisch.

Genus V. **Rhodobacillus** Molisch, 1907, p. 14

Rod shaped cells, solitary usually, motile.
The type species is *Rhodobacillus palustris* Molisch.

Genus VI. **Rhodovibrio** Molisch, 1907, p. 21.

Cells short, comma shaped, free, actively motile by means of a single terminal flagellum.
The type species is *Rhodovibrio parvus* Molisch.

Genus VII. **Rhodospirillum** Molisch, 1907, p. 24

Cells spiral, actively motile by means of polar flagella.
Rhodospirillum rubrum (Esmarch) Molisch is the type species.

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STUDIES ON PROTEOLYTIC ACTIVITIES OF SOIL MICROÖRGANISMS, WITH SPECIAL REFERENCE TO FUNGI[1]

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INTRODUCTION

It has long been recognized that when organic matter is applied to the soil it must be broken down into certain simple compounds before it can be assimilated by the roots of the plants and built up again into living tissues. This holds true, particularly, with nitrogenous compounds, such as the different proteins found in the plant residues, animal manures, and other protein substances applied to the soil. The plant is unable to assimilate the nitrogen from the complex protein bodies, and the latter must first be decomposed by means of other agencies before the assimilation of the nitrogen by the plant can take place. It is not known whether the organic and mineral acids found in the soil or those possibly excreted by the roots of the plants play any part in the hydrolysis of these proteins. But it is known that the microorganisms of the soil are able to decompose the proteins and liberate the nitrogen, which is enclosed in the complex protein molecule, in a simple form, which, either at once or after undergoing transformation due to the action of other bacteria, is easily assimilable by the roots of higher plants.

A great deal of attention has been paid by the students of soil fertility to this process of decomposition, which the organic matter undergoes in the soil; it would take up too much space to

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enumerate all the work that has been done along these lines, particularly the so-called "ammonification" studies. The fact is almost decided upon that the microörganisms decompose the organic matter in the soil, liberating ammonia, which is oxidized by other groups of organisms to nitrites, and then to nitrates, in which form it is utilized by higher plants. Several investigations seem to point to the fact that, in the absence of nitrates, other compounds, such as certain amino-acids, as shown by Skinner (1912a) and Brigham (1917), creatinine, studied by Skinner (1912b) and Brigham (1917), amides and ammonium salts, as shown by Hutchinson and Miller (1912), and other organic compounds (Brigham, 1917) can also be utilized by plants.

Very little work has been done on the process of decomposition of proteins as such, taking into consideration the intermediate steps and finding out whether the ammonia and other substances formed are merely products of metabolism or are final decomposition products. The organisms themselves must be studied, so as to learn by what biochemical processes the work is accomplished and what ends are attained, and only thus shall we be able to learn how to utilize the microörganisms for our needs in the economy of the soil and in other lines of human endeavor.

HISTORICAL

The work of Fisher and his followers, having firmly established the general structure of the protein molecule, has given a great impetus to the study of the hydrolysis and the synthesis of proteins by chemical and biological means. A great many investigations have been made of the action of bacteria and molds on proteins. It has been conceded that a great deal of this work is done by enzymes, but most of the data at hand are merely qualitative in nature, and few investigators have obtained definite quantitative information concerning the action of microorganisms or their enzymes upon protein compounds.

It has not been decided as yet, what nitrogen compounds form the *Bausteine* for these organisms. Czapek (1902) and others have shown that amino-acids form a much better source of nitro-

gen for fungi than other nitrogen compounds. Hence he argued that since the fungi must build their own proteins out of the ammonium compounds and nitrates through the amino-acid-stage, if the latter is offered to the organisms as a source of nitrogen, it is assimilated as such and the organism is spared unnecessary loss of energy, which it would have to spend in the building up of these amino-acids. Abderhalden and Rona (1905) have shown that when KNO_3, glutamic acid, and glycocoll are offered to the organisms as sources of nitrogen, the proteins that are built up will contain the same amino-acids in all cases, namely glycocoll, alanine, leucine, glutamic and aspartic acids. Hence they argued that the nitrogen building stones for these organisms must be ammonia; all the nitrogen compounds must be first split into ammonia, and out of this form of nitrogen all the proteins are built up, going through the amino-acid stage. Hagem (1910) supported the claim of Abderhalden and Rona (1905) by showing that the oxy-acids of ammonia are utilized just as well by the fungi as amino-acids; therefore he thought that in the case of amino-acids, both the nitrogen and carbon are utilized by the organism: the amino-acid is first split to ammonia and oxy-acid; the ammonia is utilized for the nitrogen needs, and the oxy-acids form a ready radical for the building up of the numerous amino-acids which go to make up the fungus protein. The oxy-acids of ammonia behave in a similar way, therefore these compounds offer a better source of food to the organism than other nitrogen compounds.

Butkewitsch (1903) identified tyrosine and leucine among the amino-acids produced by the action of *Aspergillus niger* and *Mucor stolonifer* upon peptone. Rettger and his associates (1916) have shown that bacteria are unable to attack or bring about the decomposition of proteins without the aid of enzymes or other proteolytic agents; this applies not only to the more complex proteins, like egg-albumen, but in all probability to albumoses and peptones as well. A wider range of ability to attack nitrogenous substances is observed for molds, as can be seen from the work of Czapek (1902) Emmerling (1902), Brenner (1914), and others. Sears (1916) has shown that peptone cul-

tures of most bacteria give fluctuating concentrations of amino-acids, as measured by the Van Slyke apparatus; these bodies are formed and broken down continuously by the organisms, with the exception of a few strongly proteolytic bacteria; while the ammonia gradually accumulates in the medium, the amino-acid content fluctuates daily and gradually decreases in most instances. Itano (1916) using the formol-titration method of Sörensen has shown that *B. subtilis* produced a gradual increase of formol-titrating nitrogen for a period of 240 hours. The greatest proteolysis took place toward the optimum hydrogen-ion concentration; therefore he suggested the probability that the enzyme is tryptic-like in nature; on filtering the bacterial cultures, he obtained no splitting of the peptone with the filtrate, but did obtain it with the bacterial mass.

These proteolytic changes are very important in the study of soil fertility. Schreiner and Shorey (1910) isolated from the soil, among numerous other organic compounds, the amino-acids histidine, arginine, and lysine, using a weak alkali extract of soil. That these amino-acids can be utilized by the plants as well as nitrates is seen from the work of Skinner (1912a), who has shown that histidine and arginine have a beneficial effect upon plant growth and can replace nitrates.

A great deal of work has been done on the production of ammonia from different organic compounds through the action of microörganisms, but no attempt will be made to review that work in this paper, because the study of "ammonification" is thought to be of doubtful importance, as a factor in the fertility of the soil or activities of microörganisms, when the other factors controlling these activities are not taken into consideration.

EXPERIMENTAL

Organisms used

Aspergillus niger van Tieghem. This organism was isolated on the College Farm, at New Brunswick, N. J., from a Sassafras sandy loam that was under orchard for over twenty years. Methods of isolation and the description of soils for this as well

as for the following organisms can be found in another place (Waksman, 1916).

Aspergillus ochraceus Wilhelm. This organism was isolated from a New Jersey Alloway clay soil, which was under timothy at the time of isolation.

Aspergillus fuscus Schiemann.

Aspergillus clavatus Desmasières.

Citromyces glaber Wehmer.

Penicillium chrysogenum Thom.

Actinomyces n. sp. *penicilloides* Waksman and Curtis. A full description of this organism will appear soon.

Actinomyces violaceus-ruber Waksman and Curtis.

Actinomyces diastaticus (Krainsky) Waksman and Curtis.

Bacterium mycoides Flügge, obtained from Dr. C. B. Lipman, of the University of California. All the other organisms were isolated by the writer from different soils.

Methods used

The organisms were grown on Czapek's solution composed as follows: $NaNO_3$ 2 grams, K_2HPO_4 1 gram, KCl 0.5 gram, $MgSO_4$ 0.5 gram, $FeSO_4$ 0.01 gram, cane sugar 30 grams, distilled water 1000 cc. Another medium was also used, by substituting peptone or casein for the $NaNO_3$, or for both the nitrate and cane sugar. The media were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, which were plugged and sterilized for fifteen minutes at 15 pounds pressure; they were then inoculated with the proper organisms and incubated at 28°C. At the end of the proper incubation period, the cultures were filtered through filter paper and the filtrates used for the determination of ammonia and amino nitrogen. The aeration method of Folin (1902) was used for ammonia determinations, continuing the aeration for three hours, since a shorter period of time did not liberate all the ammonia. In certain determinations, after all the ammonia had been removed by the use of Na_2CO_3, 1 gram NaOH and 10 grams NaCl were added and aeration continued for one hour more. The use of NaOH and NaCl was recom-

mended by Steel (1910) and others as a general procedure for the determination of ammonia, so as to eliminate the formation of triple phosphate crystals which are formed on adding Na_2CO_3. But Potter and Snyder (1915) have pointed out the fact that the use of NaOH will cause a great deal of the amide nitrogen to be given off as ammonia. After the ammonia was completely removed, the solution was neutralized with acetic acid, and a

TABLE 1

The formation of amino nitrogen from casein and peptone by microörganisms

Milligrams of NH_2-N per 100 cc. of solution

PERIOD OF INCUBATION	ORGANISMS USED	CASEIN	PEPTONE
days			
Control		3.14	10.15
1	*A. niger*	1.12	8.76
4	*A. niger*	0	12.12
7	*A. niger*	6.20	3.94
14	*A. niger*	7.44	5.72
1	*A. ochraceus*	2.92	7.00
4	*A. ochraceus*	7.34	9.02
7	*A. ochraceus*	9.02	7.34
14	*A. ochraceus*	12.58	8.00
1	*B. mycoides*	2.92	4.68
4	*B. mycoides*	11.84	7.34
7	*B. mycoides*	12.98	16.36
14	*B. mycoides*	12.00	13.72
1	*Act. penicilloides*	5.10	12.84
4	*Act. penicilloides*	5.64	
7	*Act. penicilloides*	11.28	
14	*Act. penicilloides*	12.58	
Control	*Trypsin, 200 mgm.*	6.16	13.60
1	*Trypsin, 200 mgm.*	26.28	22.20
4	*Trypsin, 200 mgm.*	35.04	23.60

portion equivalent to 2 cc. of the original filtrate was used for the determination of amino nitrogen. The micro-method of Van Slyke was used in all cases. Frequent blanks were made in the same manner using 2 cc. of distilled water. The determinations were carefully carried out as directed by Van Slyke (1911, 1912, 1913), the amount of amino nitrogen found in the 2 cc. portions being corrected by the use of the blank, then multiplied by 50,

thus giving the total amino nitrogen present in the 100 cc. of the filtrate.

As a preliminary experiment, two quantities of Czapek's solution were made up containing 0.5 per cent casein or peptone in place of the $NaNO_3$. The solutions were sterilized and inoculated with *A. niger*, *A. ochraceus* *B. mycoides*, *Act. penicilloides*, and trypsin. The flasks were examined at the end of 1, 4, 7, 14 days for their amino nitrogen content.

The figures in table 1 reveal marked differences in the behavior of the different organisms. There is in all of the cultures, with the exception of *Act. penicilloides*, an initial decrease in the amino nitrogen content, which completely disappeared in the case of *A. niger* growing on casein, when four days old. The subsequent determinations show an increase in the amino nitrogen. This seems to confirm the work of Sears (1916), who has shown that the bacteria will first use the amino nitrogen present in the medium and only later attack the protein and split it into amino-acids.

It was thought advisable to test a larger number of organisms on peptone alone and obtain the amino nitrogen accumulation (we can not speak of amino nitrogen production in cultures where the growing organism was present, because a constant utilization of the amino nitrogen takes place in the cultures). Czapek's solution containing 2 per cent of peptone in place of the $NaNO_3$ was used. The results are presented in table 2.

On comparing the behavior of the different organisms in their power to accumulate amino nitrogen, we are at once struck by the action of *B. mycoides* and the two actinomyces used which is so distinct from that of the molds. It is quite possible that the molds were making a much more rapid growth than the bacterium or actinomyces, the amino nitrogen formed being rapidly used up by the organism or broken down to ammonia, or that the last named organisms have a greater ability to decompose the peptone into amino nitrogen compounds. On comparing the accumulation of amino nitrogen with that of ammonia, we find that there might be an indication of the fact that a great deal of the amino nitrogen is broken down to ammonia, as is seen in the

case of *A. fuscus* and *A. clavatus*, both of which showed at the end of thirteen days the lowest accumulation of amino nitrogen and the highest accumulation of ammonia nitrogen, while *B. mycoides* and *Act. violaceus-ruber* I and II gave the highest amounts of amino- and the lowest of ammonia nitrogen. This theory does not hold true for the *Act. penicilloides* which gave the highest

TABLE 2

Amino nitrogen and ammonia accumulation by microörganisms in a 2 per cent peptone solution

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	ORGANISMS USED	NH_2-N	NH_3-N
days			
At start		40.26	0
5	*A. niger*	24.68	7.00
13	*A. niger*	29.90	77.56
5	*A. ochraceus*	34.44	4.20
13	*A. ochraceus*	42.20	56.00
5	*A. fuscus*	28.92	5.68
13	*A. fuscus*	17.90	108.36
5	*A. clavatus*	32.19	9.80
13	*A. clavatus*	23.19	103.60
5	*Citr. glaber*	40.20	11.62
13	*Citr. glaber*	48.12	48.68
5	*P. chrysogenum*	44.36	8.12
13	*P. chrysogenum*	71.10	46.76
5	*Act. penicilloides*	72.40	6.18
13	*Act. penicilloides*	149.50	42.36
38	*Act. violaceus-ruber I*	130.63	25.20
38	*Act. violaceus-ruber II*	129.25	22.60
5	*B. mycoides*	61.70	21.00
13	*B. mycoides*	119.20	22.40

amino nitrogen and at the same time a fairly high ammonia nitrogen accumulation.

It is very possible that the organisms tested differ greatly in their power to attack the peptone and in the production of different nitrogen decomposition products; such organisms as *A. niger* and *A. fussus* do not seem to be able to allow a large accumulation of amino nitrogen, and either do not split off a great deal of it or use it up, as soon as it is formed, with the production

of ammonia as a waste product; it is characteristic that these two organisms are very rapid growers and are closely allied. At the other extreme we find organisms, such as the two strains of *Act. violaceus-ruber*, *Act. penicilloides*, and *B. mycoides*, which, without producing a very strong growth upon the medium used, showed a large accumulation of amino nitrogen containing compounds with the production of a rather small quantity of ammonia. Organisms, like *Citr. glaber* and *P. chrysogenum*, seem to allow an accumulation of amino nitrogen and ammonia, but neither in a great excess.

An experiment was started using *A. niger*, for the purpose of testing the influence of sugar upon the formation of decomposition products from proteins. Czapek's solution containing 2 per cent peptone in place of the $NaNO_3$, without the sugar, was divided into two portions: to one half 3 per cent of cane sugar was added and the second half was left without sugar, so that the peptone would have to supply to the organism both nitrogen and carbon. These solutions were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, sterilized and inoculated with an approximately equal number of spores, then incubated at 28°C. At the end of every twenty-four hours one flask from each set was taken out from the incubator for the determination of amino and ammonia nitrogen. From the ninth day till the end of the experiment, after the ammonia had been expelled by the action of Na_2CO_3, the aeration was further continued upon the addition of NaOH. Two grams of Na_2CO_3 were added to 50 cc. of medium and aeration continued for three hours. After that period 1 to 2 grams of NaOH and 10 grams of NaCl were also added to the medium, and aeration continued further, for one hour, into a fresh quantity of standard 0.1 N H_2SO_4. The data obtained from these determinations are given in table 3. The second quantity of ammonia is either due to the decomposition of magnesium-ammonium-phosphate crystals that might have been formed in the medium and were not decomposed by the Na_2CO_3, or to the decomposition of the amide nitrogen present in the medium, as was pointed out before. Both of these may account for the large quantities of ammonia expelled by the NaOH; it might be

possible that the quantity of Na_2CO_3 or the period of aeration were not sufficient to expel all the ammonia, although the medium was shown to be strongly alkaline all through the aeration process, and a period of three hours was found to be sufficient.

TABLE 3*

The accumulation of amino nitrogen and ammonia by A. niger from 2 per cent peptone, in the presence and absence of sugar

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	3 PER CENT SUGAR PRESENT			SUGAR ABSENT		
	NH_2-N by Na_2CO_3	NH_2-N by NaOH	NH_3-N	NH_2-N by Na_2CO_3	NH_2-N by NaOH	NH_3-N
days						
0	0		40.60	0		40.60
1	1.00		37.74	0.84		36.01
2	1.68		31.16	1.12		32.89
3	1.68		28.60	9.10		30.03
4	2.24		27.84	11.48		32.48
5	7.00		24.94	34.72		30.16
6	12.18		25.17	49.84		33.18
7	18.62		23.45	53.90		40.04
8	28.74		22.54	70.00		32.60
9	35.60	16.90	22.27	73.62	21.14	31.98
10	48.02	17.92	19.41	76.02	25.20	30.26
11	57.24	15.26	21.13	79.94	23.80	31.68
12	71.12	14.00	20.55	87.36	23.80	36.54
13	75.60	14.00	19.18	96.04	22.40	27.98
14	78.02	16.20	17.13	100.80	23.80	29.69
15	84.00	18.20	15.98	116.06	29.40	28.55
16	89.32	19.60	18.26	129.22	30.80	21.00
17	93.28	19.60	16.54	128.94	30.60	29.50
18				129.64	38.36	21.50
19				129.92	48.02	22.34

* Certain parts of this table have been already published elsewhere (Waksman, 1917b).

The data brought out in table 3 point to a very distinct difference in the accumulation of amino and ammonia nitrogen from peptone by *A. niger* due to the presence or absence of sugar. The production of amino nitrogen is very small; there seems to be only so much of it formed as the organism needs for its development. The gradual decrease in the total amino nitrogen shows

that the peptone is broken down, a part of the decomposition products being utilized by the growing organism, while ammonia, also perhaps other nitrogenous substances, are left in the medium as waste products of the metabolism of the organism. The daily fluctuations in the content of amino nitrogen show that the splitting of the peptone may go hand in hand with the utilization of the amino nitrogen formed and its further splitting into ammonia and other products. The accumulation of the ammonia proceeds along entirely different lines than that of the amino nitrogen: while the latter gradually declines, the amount of the former gradually rises, giving at the end of 19 days as much as 50 per cent of the total nitrogen in the form of ammonia. The lack of accumulation of amino nitrogen can be explained by the fact that the organism used in this experiment grows very rapidly and probably uses all the amino nitrogen as soon as it is split off the protein molecule, or converts it into ammonia. In the case of certain other organisms, as is seen in table 2, the amino nitrogen accumulates in the medium, either due to the slow development of the organisms or to their inability to convert amino nitrogen rapidly into ammonia. The accumulation of ammonia is gradually increasing from day to day, the velocity of the reaction depending entirely on the sugar content of the medium.

Miyake (1916), using the results obtained by other investigators on bacterial activities, has shown that the processes of ammonification and nitrification are autocatalytic chemical reactions. To apply this theory to the results obtained in table 3 and also to show more clearly the difference in velocity of ammonia accumulation due to the presence or absence of sugar, the curves of autocatalysis were computed for the quantities of ammonia obtained. The tables of Dr. T. B. Robertson (1915) were used for this work, the constants having been determined from all of the observations by the method of least squares.

The curves presented in figure 1 and figure 2 are the theoretical curves obtained, while the observed quantities are given as dots. In figure 1 we can readily see that the amount of ammonia accumulated by *A. niger* from peptone in the presence of available

carbohydrates obeys the law of autocatalysis, while, in the absence of sugar, the variations are much greater, as shown in figure 2. The normal growth of the organism is disturbed in the absence of available carbohydrates when the organism has to attack the protein molecule not only for the nitrogen requirement, but also for its carbon supply. The quantities of ammonia obtained from the first to the fifth day, in the absence of sugar, are less than the calculated values, perhaps due to the fact that

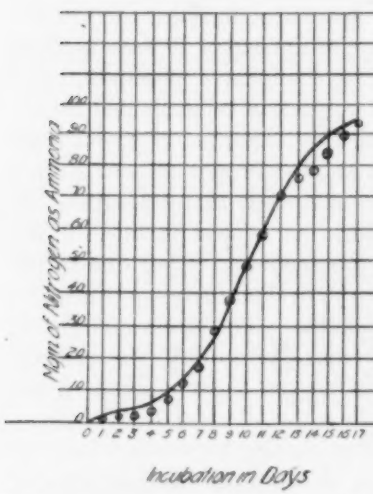

FIG. 1. AMMONIA ACCUMULATION BY *A. niger* IN THE PRESENCE OF SUGAR

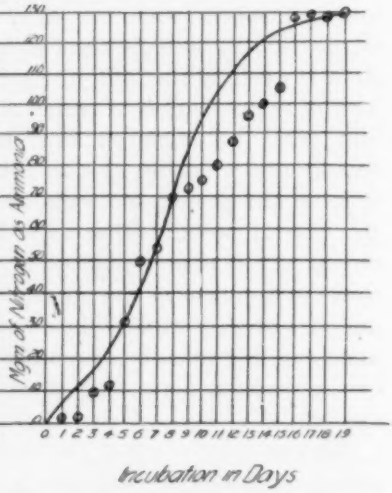

FIG. 2. AMMONIA ACCUMULATION BY *A. niger* IN THE ABSENCE OF SUGAR

it may take some time before the organism can begin to function normally when it has to derive its energy from the protein molecule; from the ninth till the sixteenth day, after the ammonia accumulation reached 70 mgm., there is another deviation of the calculated from the observed data. A similar but slighter deviation is observed also in figure 1, after the amount of ammonia reached 70 to 75 mgm. This may be either due to the exhaustion of the energy supply in the medium, or to the formation of a new autocatalytic curve. Further work on this subject continued for a much longer period of time would therefore be desirable.

As was already pointed out in another place (Waksman, 1917), the amount of available carbohydrates has a decided influence upon the metabolism of the organism and its power to accumulate ammonia from the splitting of proteins. Where sugar or other available carbohydrates are present, the organism will utilize these as a source of energy and split off from the protein only as much nitrogen as it needs for its metabolism; the quantity of ammonia accumulated in the medium will therefore be small. But, when available carbohydrates are absent, the organism will attack the protein molecule, not so much for the nitrogen as for the carbon part of it. The protein molecule decomposed into its constituent groups will lose its carbon, while only a small part of its nitrogen will be used up by the organism, because the carbon requirement of the organism is much greater than its nitrogen need, and therefore most of the nitrogen will be accumulated in the medium as a waste product, in the form of ammonia. A full discussion on this subject will be found in the article previously cited (Waksman, 1917). Doryland (1916) has shown that glucose has a detrimental effect upon ammonia production by bacteria in the decomposition of proteins; he attempted to explain this by the fact that the organisms consume some of the ammonia liberated by them from the protein, thereby leaving less ammonia in the soil or in the culture medium. Doryland assumed that the ammonia is produced by the bacteria even in the presence of glucose and is reconsumed by the organisms which are able to multiply more readily and use the more available food, although in another place Doryland himself states that "the presence of dextrose sometimes may lessen the amount of casein decomposed and amount of ammonia accumulated."

To throw more light on the production of ammonia from simpler compounds and thus perhaps indicate the possible formation of ammonia from proteins, the following experiment was started: Asparagine was added in quantities of 1, 5, 10, and 25 grams per liter of Czapek's solution, with the elimination of the $NaNO_3$. Each liter of medium was distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, and these were sterilized as usual. All the 40 flasks were inoculated with an approxi-

mately equal number of spores of *A. niger* and incubated at 28°C. At the end of twenty-four hours a flask from each set was taken out for the determination of ammonia and amino nitrogen; this was repeated at regular intervals of twenty-four hours. The data obtained are presented in table 4.

The relation between the utilization of amino nitrogen by *A. niger* and the formation of ammonia is revealed in the above table. Asparagine contains half of its nitrogen in the form of amino nitrogen, as determined by the method of Van Slyke. The

TABLE 4

The utilization of asparagine nitrogen and accumulation of ammonia by A. niger

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	0.1 PER CENT ASPARAGINE		0.5 PER CENT ASPARAGINE		1 PER CENT ASPARAGINE		2.5 PER CENT ASPARAGINE	
	NH_2-N	NH_3-N	NH_2-N	NH_3-N	NH_2-N	NH_3-N	NH_2-N	NH_3-N
days								
0	9.53	0	47.69	0	95.28	0	238.16	0
1	9.16	0	46.76	0	93.97	0	236.00	0
2	8.29	0.28	44.45	0.28	90.50	0.98	221.65	0.14
3	7.39	0.56	40.02	0.14	86.56	0.84	186.40	6.58
4	6.26	0	33.18	2.80	81.73	6.30	177.32	18.76
5	4.58	0	26.31	2.38	74.36	5.60	173.32	19.04
6	3.50	0	19.86	3.64	70.08	7.00	168.19	28.00
7	2.85	0.14	7.98	8.40	55.86	12.32	136.80	40.32
8	2.28	0	7.41	10.92	53.01	19.88	93.18	76.98
9	1.15	0	6.85	11.48	35.91	24.20	85.10	84.42
10	1.15	0	5.13	14.56	30.21	28.98	47.31	148.12

amount of sugar in all the media was 3 per cent. Where the amount of asparagine was small, only traces of ammonia were found and no autolysis was noticeable at the end of the experiment. The more nitrogen the medium contained, the heavier was the weight of the mycelium and the more autolysis it underwent after the optimum growth has been completed. After the organism made its optimum growth, the ammonia began to accumulate in the medium very rapidly. With the high concentration of nitrogen in the medium, the sugar became the limiting factor, and the rapid accumulation of ammonia was either due to the autolysis of the organism or to the breaking up of the

asparagine molecule for the utilization of the carbon part of it, thus leaving the nitrogen in the medium as ammonia.

Ammonia is usually looked upon as a final product in protein metabolism. That this is not the fact has recently been pointed out by Berman (1916), who stated that the determination of free ammonia as an index of protein metabolism should be condemned. As a matter of fact we have very little knowledge on that point. The accumulation of ammonia in the medium is probably due to several causes: firstly, it is an important waste product in the nitrogen metabolism of microörganisms; secondly, it is possible that, for certain organisms at least, the proteins and other nitrogenous substances must be first broken down to ammonia, this being assimilated as such by the organism, although we have no direct evidence on that point; in such a case the organism may split off more ammonia than it can use, so that a great deal of it is left in the medium. The fact that ammonia is not the final product of protein metabolism can be demonstrated in the case of those organisms that accumulate a large quantity of amino nitrogen, as was shown for *P. chrysogenum*, *Act. penicilloides*, *Act. violaceus-ruber*, and *B. mycoides*. Or it is possible that the different microörganisms behave differently in this respect, and the ammonia which may be only a waste product for some organisms may act as an intermediate product for others.

The problem of proteolytic activities of microörganisms is not such a simple one as might be thought at first. Most bacteriologists limit themselves to the study of one particular nitrogenous product, usually ammonia, and thus conclude that they are studying protein metabolism, often without taking into consideration the numerous controlling factors. In a number of experiments on ammonification in the soil by the so-called "beaker method," the author was able materially to alter the ammonia produced from a certain organic substance, using the same soil, the same moisture content, temperature and period of incubation, so as to have all the environmental conditions as much alike as possible, and merely changing one factor, such as the size of the soil particles: when the soil was placed in the beaker in small lumps, the greater aeration allowed a better development of the

fungi than of the bacteria; and the fungi, as was shown elsewhere (Waksman, 1916), produce as a group a great deal more ammonia than do the other microörganisms under the same conditions. Thus we see that by producing a slight change in the environmental conditions, we not only change the conditions for microorganic activities, but stimulate a change in the active soil flora.

The influence of any one factor is so important in controlling the amount of ammonia accumulated in the medium, that the ammonification studies, which occupy such an important place in many bacteriological investigations, particularly with soils, will have to be carefully revised. The very study of ammonification seems to be of doubtful importance, firstly due to the fact that all the controlling factors can hardly be taken into consideration in any one experiment (this refers particularly to mixtures of microörganisms); secondly, because it has not been proven as yet that ammonia is an end product in protein metabolism and under exactly what conditions it is formed as a waste product; thirdly, the ammonia produced by mixtures of microorganisms will be differently affected by different conditions which do not affect alike the different groups of microörganisms; and fourthly, if the production of ammonia by microörganisms is studied, it should not be studied by itself but in connection with the other activities of the microörganisms.

SUMMARY

1. Different organisms behave differently in their power to attack proteins and in the production of amino nitrogen and ammonia.

2. Most of the molds which grow very rapidly, as manifested by the increase in weight of their mycelium, allow a small amount of amino nitrogen to accumulate in the medium, while the amount of ammonia accumulated increases with the period of incubation.

3. Certain molds, particularly the slower growing ones, the actinomyces studied, and *B. mycoides* favor a large accumulation

of amino nitrogen in the medium and a comparatively smaller accumulation of ammonia.

4. The growth of *A. niger* upon a solution containing peptone shows that the amino nitrogen produced in the medium is used up by the organism, so that no great accumulation takes place. Ammonia, on the other hand, which seems to be a waste product of the metabolism of the organism, accumulates readily in the medium, particularly when the organism stops growing and begins to autolyze.

5. The presence of available carbohydrates checks the accumulation of ammonia in the medium, due to the fact that in their presence the organism uses only as much of the protein molecule as it needs for its nitrogen metabolism, and only a small quantity of ammonia will accumulate.

6. The process of ammonification, in the presence of available carbohydrates, is found to be an autocatalytic chemical reaction.

7. In the absence of available carbohydrates, the observed data deviated from the data calculated by the use of the curve of autocatalysis.

8. The study of ammonification is of doubtful importance in revealing to us the proteolytic activities of microörganisms, since the quantity of ammonia accumulated in the medium depends on a great number of controlling factors; it has not been proven as yet that ammonia is an end product of protein metabolism.

9. Asparagine nitrogen is rapidly converted into ammonia nitrogen, after the organism has made its maximum growth; but, where the amount of asparagine nitrogen is small, particularly in the presence of a comparatively large excess of available carbohydrates, no ammonia or only a very small quantity of it will accumulate in the medium.

The writer wishes to express his most sincere thanks to Dr. T. B. Robertson and Dr. C. B. Lipman, of the University of California, for reading the manuscript. The second part of this paper, dealing with the proteolytic enzymes of soil fungi and actinomyces will be published later.

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A MODIFICATION OF THE TECHNIQUE OF THE VOGES AND PROSKAUER REACTION

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Summary. The use of Syracuse watch glasses, 1 cc. of glucose potassium phosphate broth—incubated for forty-eight hours at 30°C.—and 0.5 cc. of a 45 per cent solution of sodium hydroxide, permits the development of a definite color reaction in this test in a maximum period of one and one-half hours after the addition of the latter solution.

While engaged in a study of the members of the colon group in the local water supplies a comparison was made between the methods for this test as described in Clemesha's "The Bacteriology of Surface Waters in the Tropics" and in the latest edition of "Standard Methods." Clemesha employed a medium consisting of 10 grams of peptone and 5 grams of glucose in 1000 cc. of water with the following procedure:

> The Voges and Proskauer reaction is completed by adding a few drops of a very concentrated solution of caustic potash after forty-eight hours' (or more) incubation. The red colouration nearly always appears within an hour, but in some cases it is delayed. The tubes are, however, kept for twenty-four hours after the caustic potash has been added.

On a following page of his book he wrote:

> Some care has to be exercised, as in all similar reactions, for in some cases the colour is rather faint. If, however, a very small quantity (2 or 3 drops) of a saturated solution of caustic potash is used, instead

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of a large quantity of dilute solution, the amount of fluid in the tube is not increased, and the colour very seldom fails to appear in sufficient quantities to be easily recognizable in two hours.

In the 1917 edition of "Standard Methods of Water Analysis," a medium consisting of 5 grams of Witte's peptone, 5 grams of c.p. glucose and 5 grams of dipotassium phosphate (K_2HPO_4) in 100 cc. of water, is recommended for the methyl red and Voges and Proskauer tests with the following procedure, after incubation at 30°C. for five days and after the former test has been made: "To the remaining 5 cc. of medium add 5 cc. of a 10 per cent solution of potassium hydroxide. Allow to stand over night. A positive test is indicated by an eosin pink color."

In the case of both media, after the addition of the sodium hydroxide, the tubes removed from the incubator in the afternoon were returned to it or allowed to stand in the laboratory until the morning of the following day. After a few parallel tests had been made it was observed that, with the same cultures, positive Voges and Proskauer reactions did not always develop in the two media. As a result of this disagreement a study was made of the factors which were thought to exert the greatest influence on the reaction, namely, the concentration of the sodium hydroxide solution and the optimum interval of time necessary for the development of the pink color.

Without giving the details of all the experiments it was found that the addition of 0.5 cc. of a 45 per cent solution (1 pound per liter of water) of sodium hydroxide to about 5 cc. of the media gave sharper and more distinct colors than that of 5 cc. of a 10 per cent solution. At the same time it was observed, in many of the tubes in which positive reactions developed at the end of five hours, that the pink color disappeared on standing over night; that the intensity of the pink color varied with different cultures; that the permanency of the color was usually proportional to the intensity; and that the color in the majority of cases developed in an interval of two hours after the addition of sodium hydroxide. The following table furnishes a comparison of the positive Voges and Proskauer reactions resulting from the use

of the two media with 283 strains of lactose fermenting organisms which were negative to methyl red. The development of the pink color was noted at the end of 1, 2, 5, and 20 to 25 (over night) hours after the addition of 0.5 cc. of a 45 per cent solution of sodium hydroxide to the tubes of Clemesha's medium and 5 cc. of a 10 per cent solution to the tubes of glucose potassium phosphate medium. The positive reactions are expressed in percentages of the 283 methyl red negative organisms.

From this table it is seen that the correlation percentages are much higher in the case of Clemesha's medium; that the five hour period of reaction with the sodium hydroxide is the optimum interval of time for the development of the pink color;

TABLE 1

Percentages of positive Voges and Proskauer reactions with 283 methyl red negative reactions

	TUBES			
	Hours after addition of sodium hydroxide			
	1	2	5	20-25
Glucose potassium phosphate broth, five days at 30°C	31.1	43.5	56.0	36.0
Clemesha's medium, two days at 37.5°C	66.3	70.2	79.9	70.6

and that the over night period gives erroneous results on account of the fading of the pink color. All tubes in which a noticeable pink color developed were called positive. Part of the negative tubes were colorless and part were characterized by a yellowish green color.

From the work of Harden and Walpole it appeared that oxidation plays an important part in the development of the pink color. In order to hasten the oxidation the more intimate exposure of a portion of the incubated culture appeared to be the next step in the investigation. Accordingly Syracuse watch glasses measuring 5 cm. in diameter by 8 mm. in depth were employed as containers. In such a dish 1 cc. of liquid is spread over an area of about 20 sq. cm. with a resulting depth of liquid of about

0.05 cm. After several trials a combination of 1 cc. of the culture and 0.5 cc. of a 45 per cent solution of sodium hydroxide was found to produce a deep pink color in about one hour's interval after the addition of the latter. The maximum intensity of the color developed between the first and second hour so that the readings were taken at both intervals. After the latter interval the color started to fade. The use of the Syracuse watch glasses was started in April, 1917.

Table 2 gives a comparison of the Voges and Proskauer reactions developing from the same cultures in glucose potassium phosphate broth incubated at 30°C. for five days and at 37.5°C. for two days and in Clemesha's medium incubated for two days

TABLE 2

Percentages of 262 methyl red negative cultures giving positive Voges and Proskauer reactions

	DISHES		TUBES			
	Hours after addition of sodium hydroxide					
	1	2	1	2	5	20-25
Glucose potassium phosphate broth:						
Five days at 30°C.	76	73	31	49	58	37
Two days at 37.5°C.	82	85	45	62	67	65
Clemesha's medium 2 days at 37.5°C.	88	89	65	73	83	74

at 37.5°C. The lactose fermenters employed in these tests were derived from Tapir feces, grass, and water samples.

From this table it is evident that:

1. Greater percentages of positive Voges and Proskauer reactions were obtained in the Syracuse watch glasses (columns headed "Dishes") than in the test tubes.

2. Some of the Voges and Proskauer reactions, positive in the tubes at the end of five hours, faded on standing an additional fifteen to twenty hours.

3. In the case of Clemesha's medium the percentages of positive Voges and Proskauer reactions were 89 and 83, respectively, in the Syracuse watch glasses at the end of two hours and in the test tubes at the end of five hours. With the glucose potassium phosphate medium, at both periods of incubation, the percent-

ages of positive Voges and Proskauer reactions in the two containers showed a marked disagreement.

4. Based on the number of positive Voges and Proskauer reactions, Clemesha's medium ranks first, the glucose potassium phosphate medium second when inoculated at 37.5°C. for forty-eight hours, and third when inoculated at 30°C. for five days.

In the course of this study it became evident that a considerable number of organisms would give positive Voges and Proskauer reactions with Clemesha's medium and negative results with the glucose potassium phosphate medium. Furthermore the latter, when inoculated with the same culture, did not always yield positive Voges and Proskauer reactions after incubation at 30°C. for five days and 37.5°C. for two days. In seeking an explanation for this non-agreement the influences of temperature and incubation period were studied by inoculating large quantities of glucose potassium phosphate broth and Clemesha's medium with various cultures so that portions could be removed for testing the Voges and Proskauer reactions at twenty-four hour intervals for a period of five days. It was found that: forty-eight hours' incubation was sufficient time for the development of acetyl-methyl-carbinol and a longer incubation period exerted an unfavorable influence. In general, the intensities of the colors which developed after the addition of sodium hydroxide diminished as the incubation period increased, faint and very faint pink colors appearing and shading, in some cases, to brown or yellow. At the end of forty-eight hours colors of a uniform strong pink shade appeared and doubtful colors were absent. A considerable percentage of cultures yielded positive Voges and Proskauer reactions at the end of twenty-four and forty-eight hours and negative at the end of five days. Table 3 contains the results of some tests made to illustrate the influence of the period of incubation on the development of positive Voges and Proskauer reactions. From each 100 cultures tested by the Voges and Proskauer reaction the tests indicated were obtained in the respective media at the various incubation periods.

Table 2 shows that cultures in Clemesha's medium incubated at 37.5°C. for two days yield a larger percentage of positive Voges and Proskauer reactions (see columns headed "Dish"—

two hours) than those inoculated into glucose potassium phosphate and incubated at 30°C. for five days and 37.5°C. for two days, the respective percentages being 89, 73 and 85. The data included in table 3 and other tests not included in this article show that glucose potassium phosphate broth incubated at 30°C. for forty-eight hours is superior to the same medium, and also to Clemesha's medium, when incubated two days at 37.5°C. for yielding positive Voges and Proskauer reactions.

TABLE 3

MEDIA AND TEMPERATURES	INCUBATION PERIOD IN DAYS				
	1	2	3	4	5
Glucose potassium phosphate broth:					
30°C.	82	82	82	78	67
37.5°C.	78	75	58	36	21
Clemesha's medium 37.5°C.	64	72	70		

Bacto-peptone furnished by the Digestive Ferments Company has been used entirely in the media mentioned in this article. Due to delay in obtaining other brands of peptone a comparative study could not be made in time to be included in this paper but the results will be presented later in connection with other factors influencing this reaction.

CONCLUSIONS

The use of 0.5 cc. of a 45 per cent solution of sodium hydroxide, Syracuse watch glasses, and 1 cc. of the culture results in the development of a greater number of positive Voges and Proskauer reactions in a shorter interval of time than the method given in the latest (1917) edition of "Standard Methods of Water Analysis."

An incubation period of two days at 30°C. is recommended in place of that of five days in order to obtain the maximum number of positive Voges and Proskauer reactions from glucose potassium phosphate broth. In order to avoid separate cultures for this test 1 cc. portions may be withdrawn from those incubated for the methyl red test.